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(19) (CA) **CANADIAN PATENT** (12)

(54) EUCARYOTIC CELLS, EUCARYOTIC PROTOPLASTS AND
MULTICELLULAR EUCARYOTIC LIVING ORGANISMS CONTAINING
DNA INTRODUCED BY LIPID VESICLES, PROCESS FOR THEIR
PREPARATION AND THEIR USE IN GENE PRODUCTS FOR
IMMUNIZATION AND FOR CURING GENETICALLY BASED DEFECTS

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The invention relates to eucaryotic cells, eucaryotic protoplasts and multicellular eucaryotic living organisms containing DNA introduced by lipid vesicles, which are able to express the genetic information in the form of useful gene products. The invention furthermore relates to a process for their production and their use for preparing gene products for the immunization of warm blooded creatures, e.g. mammals or human beings, and for curing genetically based defects in the above-mentioned organisms.

Lipid vesicles, sometimes also called liposomes, are approximately spherical structures. Their walls consist of a system of at least one membrane of lipid molecules. At the present time three different kinds of lipid vesicles are known, viz. multilamellar lipid vesicles (MLV), also called liposomes, small unilamellar lipid vesicles (SUV) and large unilamellar lipid vesicles (LUV). The production, the properties and the use of these lipid vesicles has, for instance, been described by G. Poste, D. Papahadjopoulos and W.J. Vail, *Lipid Vesicles as Carriers for Introducing Biologically Active Materials into Cells, Methods in Cell Biology*, Vol. 14 (1976), pp. 33 - 71, and R.E. Pagano and J.N. Weinstein, *Ann. Rev. Biophys. Bioeng.*, Vol. 7 (1978), pp. 435 - 468. These two review articles refer to numerous further literature references.

It is known that cells of warm blooded creatures, for instance mammal cells, accept in vitro and in vivo a large number of lipid vesicles without cytotoxic effects.

Since the most various of substances may be entrapped in the interior of lipid vesicles or between the vesicle membranes, the uptake by cells of lipid vesicles of a particular composition may open up a possibility for modifying the cellular composition and for introducing nonpermeable biologically active substances into the cell; see G. Poste, D. Papahadjopoulos and W.J. Vail, loc. cit., and G. Gregoriadis, The Carrier Potential of Liposomes in Biology and Medicine, The New England Journal of Medicine, Vol. 295 (1976), pp. 704 - 710, and R.E. Pagano and J.N. Weinstein, loc. cit.

It is known, for instance, to introduce inositol hexaphosphate containing unilamellar lipid vesicles into the interior of erythrocytes; see K. Gersonde and C. Nicolau, Blut, Vol. 39 (1979), pp. 1 - 7. Furthermore it is known that metaphase chromosomes carrying the HGPRT gene or the AMP-pyrophosphate-phosphoribosyltransferase gene (aprt⁺) can be introduced with liposomes into cells which are HGPRT or aprt negative. The transfer may be confirmed by selection of the few HGPRT or aprt positive cells obtained; see A.B. Mukherjee et al, Proc. Natl. Acad. Sci. USA, Vol. 75 (1978), pp. 1361 - 1365, and M. Wigler et al, Proc. Natl. Acad. Sci. USA, Vol. 76 (1979), pp. 1373 - 1376. It is also known to introduce Type I polio viruses or the RNA of these viruses which is capable of replicating, into polio virus resistant chinese hamster ovary cells with the aid of lipid vesicles. In this case the transfer is confirmed by demonstrating virus production; see T. Wilson et al, Proc. Natl. Acad. Sci. USA, Vol. 74 (1977), pp. 3471 - 3475, and T. Wilson et al,

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Cell, Vol. 17 (1979), pp. 77 - 84.

Similarly, rabbit globin m-RNA has been transferred using lipid vesicles (LUV) into the spleen lymphocytes of mice, where subsequently the synthesis of rabbit globin can be observed; see G.J. Dimitriadis, Nature, Vol. 274 (1978), pp. 923 - 924. The use of RNA does, however, have the disadvantage that it is difficult to purify and its purified state is much more readily hydrolysed than DNA.

The bacterial, self-replicating plasmid pBR322 may be introduced with the aid of liposomes into E. coli. It replicates in E. coli and its presence can be detected via the successor bacteria of the receptor bacterium, which now carry the resistance marker of pBR 322; see R.T. Fraley et al, Proc. Natl. Acad. Sci. USA, Vol. 76 (1979), pp. 3348 - 3352. Liposomes containing DNA have been introduced into the nucleus of the protoplasts of the plant Vigna sinensis; see P.F. Lurquin, Nucleic Acids Research, Vol. 6 (1979), pp. 3773 - 3784. It is further known that liposomes can be injected into living organisms and fuse with the cells of the organism.

The entrapment of plasmid DNA (pMB9) in lipid vesicles (LUV) is also known; see G.J. Dimitriadis, Nucleic Acids Research, Vol. 6 (1979), pp. 2697 - 2705.

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It is one object of the invention to provide eucaryotic cells, eucaryotic protoplasts and multicellular eucaryotic living organisms containing a single-stranded or double-stranded DNA of procaryotic or eucaryotic origin introduced by means of lipid vesicles; the aim of this is immediate gene expression, i.e. the synthesis of useful gene products in the cells and in an amount which enables said products to be detected, enriched and isolated easily. In contrast to the known processes for gene transformation, it is possible according to the invention to determine the gene products according to known methods and to obtain them without the need for selection from the rest of the cells those which have received the DNA-containing lipid vesicles. In addition to the primary useful gene products (proteins in the form of enzymes, virus antigens, peptide hormones, biologically active polypeptides, etc.), the invention also sets out to provide secondary gene products which are enzymatically synthesized by the primary gene products such as enzymes.

A further object of the invention is to provide eucaryotic cell-lines which synthesize permanently and in the form of their successor cells specific valuable gene products not only directly, but by stable incorporation of the DNA introduced by means of lipid vesicles.

A further object of the invention is to introduce DNA by means of lipid vesicles into multicellular eucaryotic living organisms without endangering their integrity

by major manipulations, and thereby to stimulate the production of certain gene products, e.g. the formation of antigens with the aim of "self-immunization", the formation of antibodies, or the formation of gene products for temporarily or permanently curing genetically based deficiencies, defects or diseases.

It is yet a further object of the invention to introduce DNA-containing lipid vesicles into the cytoplasm of eucaryotic protoplasts of plants, in order to form useful products in the plant, e.g. proteins, and after transferring the DNA into the cell nucleus to obtain genetically novel plant varieties via cellular mechanisms. It is also possible using the invention to provide *Saccharomyces cerevisiae* and *Neurospora crassa* and their protoplasts containing DNA introduced by means of lipid vesicles.

It has been found quite surprisingly that after DNA is introduced via lipid vesicles into the cytoplasm of eucaryotic cells, i.e. isolated cells of plant, animal or human origin, eucaryotic protoplasts or cells of multicellular eucaryotic living organisms,

direct production of the corresponding gene products may be found to have taken place within only a few hours. It is also surprising that after introducing DNA-containing lipid vesicles into multicellular eucaryotic living organisms, production of the corresponding gene products can be ascertained to be taking place within only a short time in the organisms. Hitherto it was not known that it was possible to express DNA from procaryotic or eucaryotic sources in eucaryotic systems

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immediately and directly after introduction of the DNA-containing lipid vesicles. The mechanism of the transcription and translation of DNA in procaryotic and eucaryotic systems has not yet been fully explained.

As eucaryotic cells for the present invention virtually all conventional cells and cell-lines of different origins may be used, such as HeLa cells (human beings), L-cells (mice), Rous Sarcom cells (chicken), Vero cells (monkey), kidney cells (monkey, cattle, pig), as well as primary cell cultures, which may be obtained directly from various organisms such as mice, rats, chickens, monkeys, quail, hamsters, rabbits, cattle or pigs. Especially preferred are diploid cells, which in tissue cultures achieve the highest possible generation numbers. Cells and protoplasts from, for instance, tobacco plants, cereal plants, *Saccharomyces cerevisiae* or *Neurospora crassa* may also be used. As multicellular eucaryotic living organisms plants, animals or human beings may be used.

Suitable DNA material from procaryotic or eucaryotic sources is biochemically purified total DNA of a cell or organism, especially enriched fractions (gene fragments). Single-stranded or double-stranded DNA, especially specific DNA, is available today after replication in plasmids, phages or viruses in any desired amounts. Plasmid DNA and virus DNA (vectors) can preferably be used, which may in turn themselves contain specific donor DNA from other organisms. This process is especially suitable because the desired DNA may thereby be enriched.

In particular gene fragments excised from the vectors may be used which contain codogenic DNA sequences for the desired gene product. It is not a necessary precondition for the invention that after fusion with the cells or cells of living organisms the DNA introduced into the lipid vesicles should be capable of self-replication. DNA may also be manufactured by means of reverse transcription from RNA. It is also possible to use chemically prepared DNA sequences either alone or after coupling to natural DNA or DNA obtained by other means, e.g. in plasmids.

The production of the DNA-containing lipid vesicles and their introduction into eucaryotic cells, eucaryotic protoplasts and multicellular eucaryotic living organisms is effected in accordance with conventional known methods. In particular, the lipid vesicles are introduced into the cells, protoplasts or organisms in accordance with the processes described by G. Poste, D. Papahadjopoulos and W.J. Vail, loc. cit., and by R.E. Pagano and J.N. Weinstein, loc. cit.. DNA-containing lipid vesicles may be administered to multicellular eucaryotic living organisms, such as animals and human beings, by parenteral injection, for example intravenously, subcutaneously, intraperitoneally or else by injection into specific organs, for example intratesticularly. DNA-containing lipid vesicles may be introduced into plants systemically into the vessels, e.g. the vascular bundles or sieve-tubes.

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After fusion with DNA-containing lipid vesicles, valuable primary gene products such as antibodies, enzymes like β -lactamase, cellular, bacterial or viral antigens like tumor antigens, H-antigens of Salmonellae, the surface antigen of the hepatitis B virus (HBsAg), antigens of the rabies virus or foot-and-mouth disease virus can be manufactured using the cells. In principle it is also possible to prepare products which are formed by consecutive synthesis of one or more enzymes whose genetic information was introduced via the lipid vesicles into the cells. The same is true of multicellular organisms whose cells have been fused with DNA-containing lipid vesicles.

As regards the cells, the following applications are possible:

- a) first and foremost the possibility of synthesizing the desired gene product immediately, without the introduced DNA having to replicate itself, and without having to wait for these cells to replicate and propagate;
- b) thanks to the high degree of efficiency of the DNA transfer via lipid vesicles cells may be isolated very easily which contain the DNA in such a way in their genome that the DNA may be transferred hereditarily and leads to continuous synthesis of the desired gene product. According to the prior art to date, i.e. in conventional transformation methods, at most one cell in around one million would be expected to contain the desired novel property.

As regards multicellular living organisms, animals, for instance, may be stimulated to form antibodies against diseases which are caused by parasites, bacteria or viruses. In the field of human medicine hereditary defects such as galactosemia, Lesch-Nyhan syndrome, maple syrup disease, hyperargininemia, hemophilia A and B or muscular dystrophy can be treated. Similarly, diseases can be treated whose therapy requires the intracellular synthesis of proteins in the organism itself. It is possible by the fusion of cells of a living eucaryotic organism with DNA-containing lipid vesicles to remedy a protein deficiency such as a peptide hormone or enzyme deficiency, or to increase defence against viral infection (by the production of interferon).

The process for preparing the DNA-containing lipid vesicles generally comprises mixing at conventional pH values, preferably a pH value of about 7, in aqueous solutions at temperatures at which the lipid vesicles are stable, preferably at around 35 to 37°C, water insoluble polar lipids (so-called amphipathic lipids), e.g. phospholipids, either alone or as a lipid mixture such as a phospholipid, frequently egg lecithin, cholesterol and an electrically charged amphiphile such as stearyl amine (positive charge) or dicetylphosphate (negative charge) in various molar ratios, most commonly in the range of 6 : 3 : 1 to 5 : 5 : 0.5, with DNA material. Subsequently lipid vesicles are formed, e.g. by ultrasonic treatment, which entrap the DNA material without requiring further treatment. In accordance with the invention

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eucaryotic cells or cell cultures or eucaryotic protoplasts are added as cultures to the DNA-containing lipid vesicle suspension obtained. These cultures are prepared in accordance with routine methods. Before the DNA-containing lipid vesicle suspension is added, the culture medium is partly or completely removed. After several hours the lipid vesicle solution is removed and new culture medium is added. After about 16 to 24 hours a sufficient amount of the desired gene product can be demonstrated in the cells or protoplasts.

As methods of demonstrating the synthesized gene products known methods may be used, such as microbiological assay, spectral photometry, radioimmunological, virological or enzymatic processes. All these methods are published in the standard literature. As methods for isolating and purifying the gene products known methods may likewise be used, for example chromatography, salting out, electrophoresis, or reaction with antigens or antibodies. These methods too are published in the scientific literature.

For the immunization of animals and human beings or for the production of antigens or antibodies the DNA-containing lipid vesicles may be administered in the form of conventional injection preparations parenterally according to conventional methods, e.g. subcutaneously, intravenously, intramuscularly or intraperitoneally. The DNA-containing lipid vesicles may also be injected directly into organs. It is obvious that in this case the production of the DNA-containing lipid vesicle preparations must occur under sterile conditions. Injection preparations contain a sufficient amount of the desired DNA-containing lipid vesicles in a conventional diluent,

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e.g. physiological saline. The DNA-containing lipid vesicle preparations may be injected in a single dose or several times at intervals of hours or days. The amount of DNA-containing lipid vesicles administered to an animal or human being is such that the desired immunization occurs or antigens or antibodies are so produced that sufficient quantities are isolated and obtained.

The examples illustrate the invention.

EXAMPLE 1

10 μ Mol of phosphatidyl choline (purified according to W.S. Singleton et al, Journal of American Oil Chemist's Society, Vol. 42 (1965) p. 53) and phosphatidyl serine of high purity from cattle brain in a molar ratio of 9 : 1 are dissolved in 10 ml of highly purified chloroform. The resulting solution is evaporated to dryness in a nitrogen stream at 36°C.

2 μ g of the gene β -lactamase (see J.G. Sutcliffe, Proc. Natl. Acad. Sci. USA, Vol. 75 (1978), pp. 3737 - 3741) are dissolved in 10 ml of an aqueous tris-histidine sodium chloride buffer (25 mMol tris-HCl), 2mMol histidine, 145 mMol NaCl; pH 7.4). The resulting solution is added to the lipids and the mixture is treated for 30 minutes at 35°C under a nitrogen blanket with a Branson ultrasonic apparatus Type B-12 in such a manner that no significant depletion of the DNA occurs.

The DNA-containing lipid vesicle suspension obtained is now incubated for 2 hours at 37°C with HeLa cells. For this purpose the HeLa cells are first washed without serum with 20 ml of Eagle's Minimal Essential Medium (EMEM).

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Then 4 ml of the DNA-containing lipid vesicle suspension (10 μ Mol) are added to 10⁷ washed HeLa cells.

After incubation the lipid vesicle suspension is decanted from the HeLa cells. The HeLa cells are washed twice with phosphate-buffered saline (PBS) and then 20 ml EMEM are added, which contains 10 % fetal calf serum.

In the same way, in place of HeLa cells chicken embryo fibroblasts and in a further experiment L-cells are used in the same amount.

The gene expression in the HeLa cells, fibroblasts and L-cells is followed spectrophotometrically and microbiologically. After incubation for 16 to 24 hours at 37°C 10⁷ cells are trypsinized in a conventional manner and then centrifuged at 270 x g. The resulting precipitate, i.e. the "pellet", is treated ultrasonically to break up the cells. Aliquots of the cell extract obtained are reacted for 30 minutes at room temperature with 10⁻⁴ molar cephalosporin 87/312 solution, i.e. a solution containing 51.6 μ g/ml of the cephalosporin (see C.H. O'Callaghan et al., Novel Method for Detection of β -Lactamases by Using a Chromogenic Cephalosporin Substrate, Antimicrobial Agents and Chemotherapy, Vol. 1 (1972), pp. 283 - 288). The change in the absorption spectra of the reaction mixtures is recorded with a Cary 17 spectrophotometer. The maximum value at 386 nm decreases and simultaneously a new maximum appears at 482. This is evidence of the presence of β -lactamase in the reaction mixture which has cleaved the β -lactam ring of the cephalosporin.

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The microbiological test for determining the presence of β -lactamase, for which the gene is coding, is carried out as follows:

10^6 ampicillin sensitive cells of *E. coli* C 600 are incubated with 0.5 ml of the cell extract for 15 to 18 hours at 37°C in the presence of 30 μ g/ml ampicillin. The ampicillin sensitive cells multiply vigorously in the medium. This is evidence of the presence of the gene product β -lactamase, which has cleaved the β -lactam ring of the ampicillin and thereby inactivated the antibiotic.

EXAMPLE 2

Example 1 is repeated, however in place of the DNA of the β -lactamase gene 2 μ g of the DNA fragment are used which contains the DNA sequences coding for HBsAg. The resulting DNA-containing lipid vesicle suspension is added to HeLa cells or chicken embryo fibroblasts.

After at least 18 hours incubation of the treated cells at 37°C the culture medium is analyzed by means of radioimmuno assay for the presence of HBsAg. The result of the radioimmuno assay shows that HBsAg is present.

The DNA fragment containing the DNA coding for HBsAg may be obtained in accordance with the method described by P. Valenzuela et al, *Nature*, Vol. 280 (1979), pp. 815 - 819.

The HBs antigen may be purified by known biochemical processes such as sedimentation or density gradient centrifugation.

EXAMPLE 3

Example 2 is repeated, however as DNA a plasmid is used which contains the gene for the hepatitis core antigen (HBcAg).

The result of the radioimmuno assay shows the presence of HBcAg.

In the same manner plasmids may be used which contain genes for growth hormones, insulin, somatostatin (chemically synthesized codogenic sequences), angiotensin II or interferon. The gene products can be detected using the radioimmuno assay or suitable biological methods after a short time.

EXAMPLE 4

Production of HBsAg in Rabbits

Lipid vesicles prepared in accordance with Example 2, containing DNA coding for HBsAg, are suspended in 4 ml of physiological saline and are injected into the vein of the rabbit ear. Up to the seventh day serum samples are taken daily. In addition animals are killed on selected days and liver analyses made. From the liver samples the cytoplasm is obtained using known methods. A radioimmuno assay shows the presence of the HBs antigen.

The main purpose of this process is generally to immunize animals against viral and other infectious diseases by the production of antigens and a subsequent immune reaction, i.e. the formation of specific antibodies.

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